A rapid and versatile method to transfer an insert between single-stranded vectors and reverse its orientation

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We report a novel method to transfer an insert from single-stranded M13mp18 (the donor) into single-stranded pUC119 (the recipient); the resultant insert orientation is opposite that found in the donor. The method is performed as follows: The single-stranded recipient vector is linearized at a specific site using an oligonucleotide and restriction enzyme. The resultant termini are annealed to sequences that flank the insert contained in the single-stranded donor vector. The annealed molecules are simply transformed into competent *E.coli* then incubated on solid media that selects for the recipient.

In routine molecular biological work, it is often desirable to reverse the orientation of an insert in a plasmid or phage vector or to transfer the insert into a different vector. The traditional method to manipulate an insert involves digesting double-stranded DNA with restriction enzymes at sites that flank the insert followed by ligation into a second vector, transformation into *E.coli*, then screening to obtain the clone of interest (1). Not only is this method somewhat time consuming, but problems often arise when appropriate restriction sites are not present. Here we report a method, explained in Figure 1, to transfer an insert between two single-stranded vectors. The vectors tested were M13mp18 as the 'donor' and pUC119 as the 'recipient' but other vector combinations should work (see last paragraph). We believe the requirements of the method are: 1) Both vectors must contain a single strand origin of replication (1, 2). 2) It must be possible to select the recipient from the donor (pUC119 encodes ampicillin resistance). 3) The donor and recipient vectors must share complementary sequences (the polylinkers), the insert must lie within or between these sequences, the recipient must be cut within the complementary sequences, and the resultant termini must anneal to regions on the donor that flank the insert of interest. A minimum or maximum number of complementary bases was not determined but we doubt the procedure will work if the insert is too close to the end of the polylinker.

The advantages of this method lie in its versatility and speed. Every insert is treated identically, regardless of what restriction sites it contains. Once a stock of linearized recipient vector is prepared, aliquots are used allowing many inserts to be transferred in a few hours; colonies are obtained the next day. If the β-galactosidase blue-white color selection is used (contained in pUC119), it is possible to visually identify the recombinant colonies. Nearly all the recombinant colonies contain the desired construct so a screening stop is often unnecessary. This method is also useful to reverse inserts because the orientation of the insert in the resultant recipient is opposite that of the donor.

Single-stranded DNA was isolated and purified by the addition of M13KO7 helper phage as described previously (1) with the following additions. The culture supernatant was transferred to a new tube and recentrifuged to remove residual cells, the phenol extraction was followed by a chloroform extraction, and after the final 70% ethanol wash, the samples were recentrifuged and the residual liquid carefully drawn off. Special care was taken to obtain pure template.

To linearize single-stranded DNA at a specific restriction site (3), an oligonucleotide is annealed to this site, then the resultant short region of double-stranded DNA is digested with the appropriate restriction enzyme. Only the site where the oligonucleotide hybridizes forming double-stranded DNA will be digested, even if other single-stranded restriction sites exist. Some restriction enzymes will digest single-stranded DNA to a limited extent (4) and may not be useful. Single-stranded pUC119 DNA was linearized by annealing to one of the following two oligonucleotides

HincII 5'TCTAGAGTCGACCTGCAG 3'
BamHI 5'TACCCGGGGATCCTCTAG 3'

in the appropriate restriction enzyme reaction buffer at 55°C for 30 min, then adding at least five fold excess of the appropriate restriction enzyme and incubating at 37°C for two hr. Because the recipient vector must be digested to near completion to reduce non-recombinant transformants, a molar excess of oligonucleotide is required. We found both four and forty fold molar excess of oligonucleotide led to complete digestion, but a 400 fold molar excess appeared inhibitory. The oligonucleotides should be long enough to yield efficient digestion but not so long that the resultant oligonucleotide fragments remain bound. Once linearized, the DNA was purified by phenol and chloroform extraction followed by ethanol precipitation with 0.3 M sodium acetate at room temperature. To assay the extent of linearization, the digested DNA along with undigested DNA were electrophoresed on a 1×TBE 1% agarose gel at relatively low voltages; the linearized DNA migrated slightly slower.

We used the *E.coli* strain DH5αF′ in all the experiments. Other cell types were not tested. Success of the method may be dependent on the specific cell type used. Frozen competent DH5αF′ cells were prepared as described (1) except the first resuspension of the cell pellet in FSB and the following recentrifugation was skipped (steps 7–9 on pp 1.77). The mechanism by which the cell accomplishes the transfer of the insert is not known, but might be interesting to study.

The actual transfer of the insert is done as follows: Mix a total of 5–10 ng of single-stranded DNA in a one to one molar ratio of donor and linearized recipient in one well of a 96-well microtiter plate (Costar cat. # 3799). We used (approximately) 2 ng of pUC119 and 5 ng of M13mp18 + insert. Bring to 4 μl

total 1×SSC. Add water to some empty wells if available and seal microtiter dish with plastic wrap to reduce evaporation. Incubate at 55°C for at least 30 min, then place on ice in 4°C room for at least 10 min. Thaw competent cells on ice, resuspend gently, then add 80 μl to each well containing DNA. Incubate on ice 30 min, float plate in 42°C water for 90 sec, then return to ice. Spread cells from each well on separate 2YT petri dish containing ampicillin, IPTG, and XGAL. Incubate at 37°C overnight.

Two experiments were performed to test the method (see table 1). The total yield of white colonies is about 1000 fold less than if the insert transfer were done with double-stranded DNA (data not shown). The lower yield is probably due to the lower transformation efficiency of single-stranded DNA versus double-stranded DNA. (We typically obtain 3×10^8 transformants per μg double-stranded pUC119 DNA but only 1.5×10^6 transformants per μg single-stranded pUC119 DNA.) Still, using frozen competent DH5αF′ cells, a sufficient number of recombinants from every clone (at least one) was obtained. In the second experiment the sequence of the entire insert and both vector-insert junctions was determined; in every case the sequence was perfectly conserved (data not shown).

The fate of the M13 donor DNA after transformation is not known but it is not detected by sequencing. M13 is associated with some blue and some white colonies because these colonies generate plaques when streaked on a freshly plated lawn of DH5αF′ cells, However, if a colony is first purified by streaking for isolated colonies, the subsequent colonies never produce plaques. This indicates the M13 is eventually lost.

In early attempts to perform this method, an *in vitro* polymerization and ligation step (5) was employed subsequent to the annealing of the donor and recipient and prior to transformation. This variation produced 10–20 fold more recombinants than without it and has also been successful in transferring an insert of 5 kb (an insert size limit of the '*in vivo*' variation has not been determined), but it is more labor intensive and *in vitro* polymerization reactions are error prone. Another variation of this method involved isolating linearized single-stranded DNA from undigested circular single-stranded DNA on a 1×TBE 1.5% low melting temperature agarose gel. This was successful in eliminating the background of blue colonies but only worked when the *in vitro* polymerization reaction was carried out.

This method should be of general applicability, although we have only used the vectors M13mp18 and pUC119. A vector combination that should work is Stratagene's Phagescript as the donor and pBluescript II KS (+) as the recipient. The polylinkers are longer in these vectors (112 bp vs. 60 bp for mp18 and mp19), so more sites are available for cloning and the annealed regions are longer which may improve efficiency. A vector combination that may work is Phagescript as the donor and pBluescript SK (−) as the recipient. The homologous sequences are the entire Lac IZ region. A potential advantage is a single stock of linearized recipient could be made by digesting at the two terminal sites of the polylinker (using two oligonucleotides, SacI, and KpnI). Then this stock could be used for all subsequent reactions, regardless of the site of insertion in the polylinker. Two different plasmids may also work as donor and recipient if they contained different antibiotic resistance markers, although we cannot suggest specific vectors. This method probably would not work well if M13 is used as the recipient because the helper phage used to generate the single-stranded donor would produce clear plaques making it difficult to isolate the desired construct.

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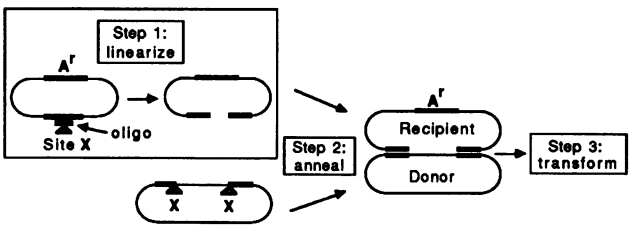

Figure 1. Method to transfer an insert between single-stranded vectors and reverse its orientation. 1) An oligonucleotide is annealed at restriction site X, then digested with the corresponding restriction enzyme. If a stock of linearized recipient is made, then aliquots can be used for future experiments. 2) The termini of the recipient vector are annealed to sequences on either side of the insert contained in the donor molecule. 3) The annealed molecules are transformed into competent *E.coli* and the recipient selected by resistance to antibiotic A.

		Colonies		Insert Size		Sequence Analysis	
		Blue	White	Correct	Incorrect	Correct	Incorrect
Expt. 1	No Donor	59	0	2	0	1	0
	Clones 1–7	35–71	8–52	22	1	7	0
Expt. 2	No Donor	~700	0	ND	ND	ND	ND
	Clones 1–18	~400	25*–120	ND	ND	15*	0

Table 1. Tests of the insert transfer and reversal method. The donor clones contained either 1.5 kb inserts in the BamHI site (Expt. 1) or short (20–500 bp) lambda fragments in the HincII site (Expt. 2) of M13mp18. The number of blue colonies (those which potentially contain parental recipient clones) and white colonies (those which potentially contain the desired recombinant recipient clones) obtained upon transformation of the annealed molecules are shown. The range of values indicates only the highest and lowest values obtained from the entire group of donor clones. Insert size indicates results of gel electrophoresis analysis (1×TBE, 0.8% agarose gel) to determine if the single-stranded DNA, produced from a culture of a single white colony, is of the correct size. Sequence analysis indicates if the above DNA contains the desired insert in the expected orientation. * = three clones that failed to produce white colonies are not shown. Sequence examination revealed the opposite orientation of two inserts would leave the reading frame intact and thus produce blue colonies. We are unable to determine why no white colonies were obtained from the third clone. ND = not done. ~ = approximately.